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Activation of p53 Sequence-Specific DNA Binding by Acetylation of the p C-Terminal Domain

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Summary

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The tumor suppressor p53 exerts antiproliferation effects through its ability to function as a sequence-specific DNA-binding transcription factor. Here, we demonstrate that p53 can be modified b acetylation both in vivo and in vitro. Remarkably, the site of p53 that is acetylated by its coactivator, p300, resides in a C-terminal domain known to be critical for the regulation of p53 DNA binding. Furthermore, the acetylation of p53 can dramatically stimulate its sequence-specific DNA-binding acti possibly as a result of an acetylation-induced conformational change. These observations clearly indica novel pathway for p53 activation and, importantly, provide an example of an acetylation-mediated cha in the function of a nonhistone regulatory protein. These results have significant implications regarding molecular mechanisms of various acetyltransferase-containing transcriptional coactivators whose prim targets have been presumed to be histones.

Introduction

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The p53 tumor suppressor protein exerts antiproliferation effects, including growth arrest and apoptos in response to various types of stress (Gottlieb and Oren, 1996; Ko and Prives, 1996; Levine, 19). The biochemical activity of p53 that is required for tumor suppression, and presumably the cellula response to DNA damage, involves the ability of the protein to bind to specific DNA sequences and tc function as a transcription factor (El-Deiry et al., 1992; Funk et al., 1992; Pietenpol et al., 1994 The importance of the DNA-binding property of p53 is underscored by the fact that the vast majority c p53 mutations derived from tumors usually map within the domain for sequence-specific DNA binding (Hollstein et al., 1991; Ko and Prives, 1996). Overexpression of p53 in cells clearly activates, through consensus p53 binding sites, a number of genes that have been implicated as functional targets p53-induced cell growth repression or apoptosis. These include GADD45 (Kastan et al., 1992), md (Barak et al., 1993; Wu et al., 1993), WAF/p21/CIP1 (El-Deiry et al., 1993), cyclin G (Okam and Beach, 1994), IGF-BP3 (Buckbinder et al., 1995), and Bax (Miyashita and Reed, 1995).

Regulation of p53 function remains less well understood. p53 is a short-lived protein that is maintainec low, often undetectable levels in normal cells (Ko and Prives, 1996; Levine, 1997). Tight regulat of p53 seems critical for normal cell growth and development, as the activity of p53 is dramatically elevated in the cells following DNA damage or during spermatogenesis (Gottlieb and Oren, 1996). precise mechanism by which p53 is activated by cellular stress is of intense interest. Although it could involve an increase in the p53 protein level, it is generally thought to involve mainly posttranslational modifications of p53 (Kastan et al., 1991; Ko and Prives, 1996). For example, at low doses of U irradiation, p53-dependent transcription is activated without any detectable increase in the p53 protein level (Hupp et al., 1995). Posttranslational modifications of the carboxyl terminus of p53 have been shown to play an important role in controlling p53-specific DNA binding (Hupp et al., 1992; Meek, 1994; Hansen and Oren, 1997; Levine, 1997). Thus, modification of a highly basic region witl the carboxy-terminal 30 amino acids of the p53 by phosporylation, antibody binding, or deletion of thi region can convert p53 from an inert to an active form for DNA binding (Ko and Prives, 1996). Furthermore, p53 C-terminal peptides stimulate DNA binding of full-length p53 in *trans* (Hupp et al., 1995; Jayaraman and Prives, 1995; Lee et al., 1995; Selivanova et al., 1997). These observations, which have led to a model for allosteric regulation of p53, are consistent with the exister of distinct cellular signaling pathways capable of modulating the conversion between latent and activat forms of p53 (Hupp and Lane, 1994).

Recently, we and others have demonstrated that CBP/p300, which also is implicated in cell proliferatic and differentiation (reviewed in Shikama et al., 1997), acts as a coactivator for p53 and potentiates transcriptional activity in vivo (Gu et al., 1997; Lill et al., 1997). The N-terminal activation dom of p53 physically interacts with the carboxy-terminal portion of the CBP protein both in vitro and in vi In transfected SaoS-2 cells, CBP/p300 dramatically potentiates activation of a p53 target gene. Since sequence-specific transcriptional activation by p53 correlates well with the ability of p53 to suppress c growth (Crook et al., 1994), the cell growth suppression function of CBP/p300 may act, at least in part, through synergistic activation with p53 on its target genes.

CBP and p300 exhibit strong sequence similarity and similar functions, such as binding to common DN binding transcription activators that include nuclear hormone receptors (Chakravarti et al., 1996; Kamei et al., 1996), CREB (Chrivia et al., 1993; Kwok et al., 1994; Arany et al., 1995; Lundblad et al., 1995), c-Jun/v-Jun (Arias et al., 1994; Bannister and Kouzarides, 1995), c-Myb/v-Myb (Dai et al., 1996; Oelgeschlager et al., 1996), Stat-1 (Zhang et al., 1996), Stat-2 (Bhattacharya et al., 1996), c-Fos (Bannister and Kouzarides, 1995), MyoD (Yuan et al., 1996) and NFκB p65 (Gerristen et al., 1997). Although the precise mechanisms by which these activators stimulate the transcriptional machinery through CBP/p300 remain unclear, recent discoveries that p300/CBP and an interacting factor (P/CAF) both have histone acetyltransferase activities (Bannister a Kouzarides, 1996; Ogryzko et al., 1996; Yang et al., 1996) suggest that CBP/p300 may play a distinct role in transcriptional regulation through histone acetylation.

Acetylated histones are a characteristic feature of transcriptionally active chromatin. Hyperacetylated histones accumulate within particular active chromatin domains, whereas hypoacetylated histones accumulate within transcriptionally silenced domains (reviewed in Turner, 1993; Brownell and Allis 1996; Wolffe and Pruss, 1996). Thus far, all core histone proteins can be variably acetylated by a number of proteins, designated histone acetyltransferases (HATs), that include GCN5, P/CAF, p300/C and $TAF_{II}250$ (Bannister and Kouzarides, 1996; Kuo et al., 1996; Mizzen et al., 1996; Ogryzl et al., 1996; Yang et al., 1996). Importantly, a global increase in core histone acetylation does nc necessarily induce widespread transcription. In some instances, an increase in histone acetylation correlated with a decrease in transcriptional activity (reviewed in Pazin and Kadonaga, 1997), suggesting that the relation between acetyltransferase function and transcriptional activity may be mor complex than a matter of histone acetylation and consequent transcriptional activation. Indeed, the possibility that these acetyltransferase activities might have other protein substrates has not been repor

In support of this possibility, we demonstrate that p300 can directly modify the C-terminal domain of p by acetylation and, remarkably, that this modification not only is position-specific, but also dramaticall stimulates the sequence-specific DNA-binding activity of p53. This discovery has important implicatio for current views regarding the molecular mechanisms by which acetyltransferase-containing coactivat effect transcriptional activation.

Results

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Identification of p53 as a Bona Fide Substrate for p300 Acetyltransferase

To test whether p53 could be specifically acetylated by p300, the following proteins were expressed w N-terminal Flag epitopes in bacteria and purified to near homogeneity on M2-agarose affinity columns region of p300 (residues 1195–1810) containing the HAT domain (Ogryzko et al., 1996), full-lengt p53 (Gu et al., 1997), Max (Blackwood and Eisenman, 1991), USF (USF-1) (Gregor et al., 199 , and the p300/CBP-interacting CREB (Chrivia et al., 1993). These highly purified recombinant proteins were used in the protein acetyltransferase assay to avoid possible contamination by either hist or other acetyltransferases.

Individual reactions containing 1 μg of each substrate were incubated with [^{14}C]-acetyl-CoA and 100 of recombinant p300 for 1 hr at 30°C. The products were analyzed by autoradiography of acetylated reaction products resolved on SDS–PAGE gels. As shown in Figure 1A, the p53 protein was specifica labeled by [^{14}C]-acetyl-CoA, whereas no signals were detected in reactions containing USF, Max, or CREB. Labeling of p53 was completely dependent on the presence of both [^{14}C]-acetyl-CoA and p30 (Figure 1B), thus excluding the possibility that the labeled p53 resulted from either a nonspecific interaction between p53 and [^{14}C]-acetyl-CoA or p53 autoacetylation.

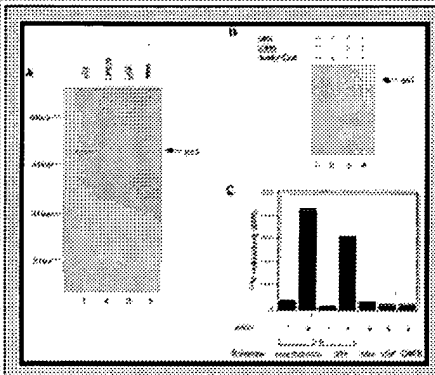

Figure 1. In Vitro Acetylation of p53 by p300 Acetyltransferase

(A) Acetylation of p53 relative to other activators. Following incubation of purified activators (1 μg) with [^{14}C]-acetyl-CoA and 100 ng of recombinant p300(1195–1810) for 1 hr at 30°C, reactior products were separated by SDS–PAGE and visualized by autoradiography. Lane 1, p53; lane 2, CREB; lane 3, USF; lane 4, Max.

(B) Substrate and enzyme requirements. Reaction products were separated by SDS-PAGE and visualized by autoradiography following incubation of various combinations of p53 (1 μg), p300(1195–1810) (100 ng), and [^{14}C]-acetyl-CoA. Lane 1, p53; la 2, p53 and [^{14}C]-acetyl-CoA; lane 3, all components; lane 4, p300(1195–1810) and [^{14}C]-acetyl-CoA.

(C) Comparative acetylation of p53 and core histones. Amounts (1 μg) of human core histones, p53, Max, USF, and CREB were incubated with [^{14}C]-acetyl-CoA in the presence or absence of p300(1195–1810). The [^{14}C]-acetate incorporation of each protein was measured by the filter binding assay.

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Furthermore, by measuring [^{14}C]-acetate incorporation in a filter binding assay (Ogryzko et al., 1996 we not only confirmed that p53 can be acetylated by p300, but also that the acetylation of p53 is as str as the acetylation of core histones by p300 under the same conditions (Figure 1C). Thus, we have demonstrated that p53 is a novel, and genuine, substrate for the p300 acetyltransferase in vitro.

The p53 Site Acetylated by p300 Is Located at the C-Terminal DNA-Binding Regulatory Doma

The p53 protein can be roughly divided into three distinct functional domains (Ko and Prives, 1996 ▣): an amino-terminal domain that contains the transcriptional activation domain (residues 1–43), a centra core that contains the sequence-specific DNA-binding domain (residues 100–300), and the multifuncti carboxy-terminal domain (residues 300–393) (Figure 2A). To map the p300-dependent acetylation site p53 in vitro, GST-p53(1–73), GST-p53(100–290), and GST-p53(290–393) fusion proteins were expressed in bacteria and purified to near homogeneity on glutathione-agarose beads (Figure 2D). As indicated in Figure 2B, only GST-p53(290–393) was detectably acetylated by p300, suggesting that th acetylation site is located in the C-terminal domain.

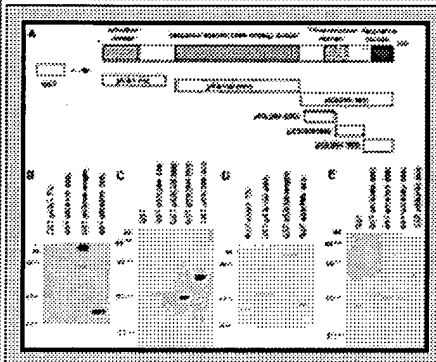

Figure 2. The p53 Acetylation Site Is Located at the C-Terminal DNA-Binding Regulatory Domain

(A) shows schematic representations of p53 and its functional domains. The p53 fragments indicated (A) were expressed as GST fusion proteins, purified to near homogeneity, and analyzed by resolution on SDS–PAGE gel and Coomassie blue staining (D and E). Corresponding GST fusion proteins (2.5 μg) were assayed for acetylation by p300 as described in Figure 1A, with reaction products analyzed by SDS–PAGE and autoradiography (B and C).

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The p53 carboxyl terminus can be further subdivided into three regions, which include (Figure 2A) a flexible linker (residues 300–320) that connects the DNA-binding domain to the tetramerization domai the tetramerization domain itself (residues 320–360), and, at the extreme carboxyl terminus, a 30 amin acid stretch that plays a very important role in the regulation of p53 DNA binding (residues 363–393). Purified GST fusion proteins containing these subdomains (Figure 2E) were tested for acetylation by p300. Although each subdomain contains several lysines, which are potential acetylation sites for acetyltransferases, only the C-terminal 30 amino acid domain was acetylated (Figure 2C).

p53 Is Acetylated In Vivo

p53 is a short-lived protein that is maintained at low, often undetectable, levels in normal cells (Ko and Prives, 1996). Compared with histones, it is extremely difficult to purify sufficient p53 protein from normal cells to test whether acetylated p53 exists in vivo. Furthermore, overexpression of wild-type p53 induces apoptosis in many cell types, thus making it impossible to obtain p53–stably transfected cell lir for this purpose.

Here, we tested whether p53 is acetylated in transiently transfected cells. As indicated in Figure 3A, Figure 3p53 can be transiently overexpressed shortly after transfection (Figure 3A, lane 2). These cells were immediately labeled by [^{3}H]-sodium acetate (1 mci/ml) for only 1 hr to limit the protein labeling t posttranslational modifications. Figure 3B shows that the p53 protein immunopurified under very high stringency conditions (see Experimental Procedures) by anti-p53 monoclonal antibody (D0-1) from th p53-transfected cell extract (Figure 3B, lane 2) was specifically labeled by [^{3}H]-acetate. No signal was detected either in proteins affinity-purified from the same extract by anti-hemagglutinin (HA) control antibody (Figure 3B, lane 1) or in proteins immunoprecipitated with the same p53 antibody from nontransfected SaoS-2 cells that were similarly labeled with [^{3}H]-sodium acetate (Figure 3B, lane 4). Interestingly, a C-terminal truncated p53 (p53Δ370), which lacks the last 24 amino acids of the protei (Crook et al., 1994; Marston et al., 1994), is expressed at comparable levels in the cells by transi transfection (Figure 3A, lane 3); however, the signal of acetylated p53Δ370, purified by the same met as acetylated wild-type p53, is significantly reduced (Figure 3B, lane 3), suggesting that the C terminu p53 may be mainly responsible for p53 acetylation in vivo. This also provides evidence indicative of similar sites of acetylation in vivo and in vitro.

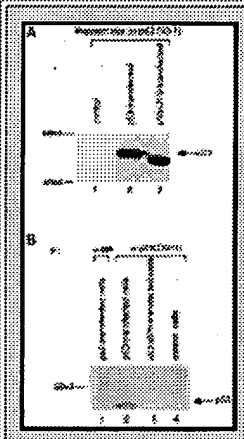

Figure 3. In Vivo Acetylation of p53

(A) Ectopic expression of p53 and p53Δ370 in Saos-2 cells. Extracts from contro cells (lane 1), p53-transfected cells (lane 2), or p53Δ370-transfected cells (lane 3) were subjected to SDS–PAGE and a Western blot analysis with anti-p53 monoclonal antibody DO-1.

(B) Radiolabeling of ectopic p53 and p53Δ370 by [^{3}H]-acetate. Extracts from control cells (lane 4), p53-transfected cells (lanes 1–2), or p53Δ370-transfected cells (lane 3) pulsed with [^{3}H]-acetate were subjected to immunoprecipitation witl anti-p53 monoclonal antibody (DO-1) (lanes 2–4) or anti-HA monoclonal antibod (lane 1), and immunoprecipitated proteins were analyzed by SDS–PAGE and autoradiography.

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Taken together, these results indicate that p53 is indeed acetylated in vivo. However, under the condit employed, the proportion of acetylated p53 remains low. This may reflect the action of strong deacety activities during the purification or rapid turnover of the acetyl residues in vivo (Hendzel et al., 1991 ; Kijima et al., 1993 ; Vettese-Dadey et al., 1996) and, to date, has limited further identification of vivo acetylation positions.

The Acetylation Site of p53 Is Evolutionarily Conserved

Recent studies have demonstrated position-specific acetylation of histone H4 by distinct acetyltransfer (Brownell and Allis, 1996). Thus, while cytoplasmic acetyltransferases for histone deposition and chromatin assembly modify positions 5 and 12, the transcriptional coactivator GCN5 preferentially modifies positions 8 and 16 (Kuo et al., 1996). To similarly determine the positions of p53 acetylate by p300, a synthetic peptide that contains the carboxy-terminal 26 amino acids was incubated with p3(and [^{14}C]-acetyl-CoA. The acetylated peptide was purified and subjected to amino-terminal peptide sequencing. The amount of [^{14}C]-acetate incorporated in each residue indicated the degree of acetylat of this residue. As shown in Figure 4A, the [^{14}C]-acetate incorporation levels for various positions indicate two peaks located at K373 and K382, respectively, suggesting that these two lysines are preferentially acetylated. Lysines K370, K372, and K381 also showed significant, but lower, levels of acetylation, whereas K386 appeared completely unacetylated in comparison with the background. The results indicate that p53 acetylation is highly position-specific.

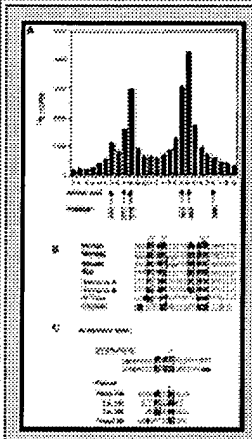

Figure 4. Identification and Evolutionary Conservation of the Acetylation Site of p53

(A) A synthetic peptide (5 μg) corresponding to the carboxy-terminal 26 amino acids of p53 was incubated with 500 ng of p300(1195–1810) and [^{14}C]-acetyl-C(for 2 hr at 30°C. The peptide was purified and subjected to N-terminal peptide sequencing (X axis; single-letter amino acid code). The amount of [^{14}C] incorporation in each residue (CPM) was determined.

(B) Alignment of the C-terminal regions of p53 from human (residues 367–388), monkey (residues 367–388), mouse (residues 361–382), rat (residues 365–386), Xenopus laevis gene A (residues 334–355), and gene B (residues 333–354), rainbow trout (residues 367–388), and chicken (residues 345–365).

(C) Comparison of the acetylation sites in human p53 with those in yeast histone H3 (residues 7–17), yeast histone H4 (residues 1–12), and Tetrahymena (Tet.) histone H4 (residues 1–10 in the upper peptide and residues 9–18 in the lower peptide). The asterisk indicates the preferentially acetylated residue.

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p53 is an evolutionarily conserved protein, particularly among vertebrate species (Soussi et al., 1990 ⊡ There is one conserved region in the amino-terminal activation domain, and there are four conserved regions in the sequence-specific DNA-binding domain, while no conserved region has been found in th terminus (Soussi et al., 1990 ⊡; Ko and Prives, 1996 ⊡). Surprisingly, a comparison of the acetylatio site of human p53 with the same region from other vertebrates reveals that the positions of all of the acetylated lysines are highly conserved, including K370, K372, K373, K381, and K382 (Figure 4B), whereas the nonacetylated K386 is not conserved (Figure 4B). These results suggest that p53 modifications by acetylation may remain highly conserved throughout evolution. Interestingly, an argi (R379) and its position are also highly conserved, indicating that besides lysine, arginine may also play role in acetylation.

p300 can acetylate all four N-terminal lysines (K5, K8, K12, and K16) in histone H4. However, differential acetylation of these sites has not been observed, perhaps due to the specific experimental approach (Ogryzko et al., 1996 ⊡). We find that five lysines of p53 (K370, K372, K373, K381, and K382) can be acetylated by p300 (Figure 4B), and that K373 and K382 are preferentially acetylated o basis of the incorporated [^{14}C]-acetate activities. In comparison with the acetylated sites identified fro the GCN5-mediated acetylation of histone H4 by a similar approach (Kuo et al., 1996 ⊡), we note tha both the pattern and the position of the preferentially acetylated lysines are very similar (Figure 4C). Taken together, these results indicate that the acetylation site of p53 is not only specific, but also evolutionarily conserved.

Acetylation of p53 Activates Its Sequence-Specific DNA Binding

A number of studies indicate that the C-terminal 30 amino acids of p53 play a key role in the regulatio sequence-specific DNA binding by p53 (Hupp et al., 1992 ⊡; Hupp and Lane, 1994 ⊡; Meek, 1994 ⊡ Jayaraman and Prives, 1995 ⊡). Since the acetylation site was mapped to this domain, it was importar examine the effect of acetylation on site-specific DNA binding by p53. To exclude the possibility that other modifications, such as phosphorylation (Meek, 1994 ⊡) or glycosylation (Shaw et al., 1996 ⊡), may interfere with this effect, we used highly purified, bacterially produced full-length human p53 in th assay. As reported previously (Hupp et al., 1992 ⊡), bacterially produced p53 is virtually inactive in site-specific DNA binding in an electrophoretic mobility shift assay (Figure 5A, lane 2 versus lane 1), t the binding can be strongly and selectively activated by anti-p53 monoclonal antibody PAb421 (Figure 5A, lane 5) relative to anti-p53 monoclonal antibody DO-1 (Figure 5A, lane 6), which nonetheless recognizes the whole p53/DNA complex, and other control monoclonal antibodies (Figure 5A, lanes 3 and 4).

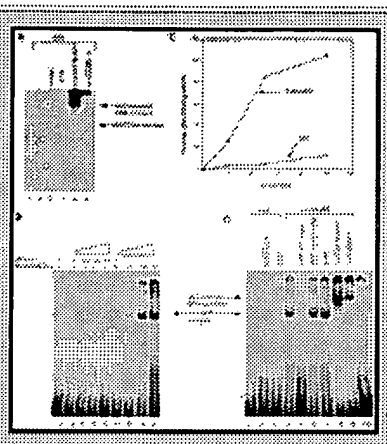

Figure 5. The Latent Sequence-Specific DNA-Binding Activity of p5 Is Activated by Acetylation

(A) Activation of bacterially produced p53 DNA binding by anti-p53 monoclonal antibody PAb421. Human p53 (50 ng, lanes 2–6) was incubated with 0.2 ng of a ^{32}P-labeled p53-binding oligonucleotide (GADD45, Kastan et al., 1992) in the absence of other proteins (la 2) or with the addition of monoclonal antibodies specific for c-Myc (lane 3), the hemagglutinin antigen (lane 4), or p53 (PAb421 in lane 5 and DO-1 in lane 6). The reaction mixtures were analyzed by EMSA. Lane 1 shows an analysis of the DNA probe without protein. The arrows indicate p53-antibody/DNA complex (upper) and p53/DNA complex (lower), respectively.

(B and C) DNA binding of unmodified versus acetylated forms of p53 Indicated quantities of unmodified p53 (lanes 2–5) and in vitro-acetylated p53 (lanes 6–9) were incubated in reaction mixtures containing ^{32}P-labeled wild-type GADD45 oligonucleotides (0.2 ng), resolved by electrophoreses, and visualized by autoradiography (B). T resulting protein–DNA complexes were quantitated (C) by phosphorimaging using Image-Quant software.

(D) Specificity of acetylated p53 binding to DNA. Recombinant p53 (ng) was incubated with ^{32}P-labeled wild-type GADD45 oligonucleotides (0.2 ng) following prior incubation with no factors (lane 1), or 1 mM acetyl-CoA (lane 2), or 5 ng p300 (lane 3). Acetylated p53 (25 ng) was incubated with ^{32}P-labeled wild-type GADD45 oligonucleotide (0.2 ng) following prior incubation with no factors (lane 4), a 200-fold excess of unlabeled wild-type (lane 5) or mutant (lane 6) GADD45 oligonucleotides, anti-HA monoclonal antibody (lane 7), anti-p53 monoclonal antibody PAb421 (lane 8), or anti-p53 monoclonal antibody (DO-1) (lane 9). Lane 10 contained the DNA probe without protein. The arrows indicate the p53-antibody/DNA complex (upper) and the p53/DNA complex (lower), respectively.

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Surprisingly, a dose response analysis showed that acetylation by p300 in vitro could dramatically incr the DNA-binding activity of fixed amounts of p53 (Figure 5B). As indicated in Figure 5C, activation o the DNA binding by acetylation reached levels as high as 20- to 30-fold over untreated p53 binding. N change in DNA-binding activity was found after treatment of p53 with acetyl-CoA or p300(1195–181 alone (Figure 5D, lanes 1–3), indicating that the stimulation of DNA binding is specifically due to the acetylation of p53. Furthermore, the strongly enhanced DNA-binding activity of p53 is sequence-speci since the binding was competed by a 200-fold molar excess of an unlabeled oligonucleotide containing wild-type p53 binding site but not by comparable amounts of an oligonucleotide containing a mutated binding site (Figure 5D, lanes 4–6).

Interestingly, the acetylated form of p53 was supershifted both by anti-p53 monoclonal antibody PAb4 (epitope in the C-terminal domain of p53) and by anti-p53 monoclonal antibody DO-1 (epitope in the N-terminal domain of p53) (Figure 4D, lanes 8 and 9), similar to what was observed with the unmodifi form (Figure 4A, lanes 5 and 6). Whereas DO-1 did not enhance binding either of acetylated p53 (Fig 4D) or unmodified p53 (Figure 4A), PAb421 did enhance binding of acetylated p53 (Figure 4D). However, this latter effect was minimal (~2-fold) compared to the very large effect (over 20-fold) of PAb421 on DNA binding by the unmodified p53 (Figure 4A). This suggests that activation by anti-p5 antibody PAb421 and activation by acetylation may involve similar molecular mechanisms.

Insight into the Mechanism of Acetylation-Mediated Activation

The specificity of the effect of the p53 C terminus on p53 sequence-specific DNA binding suggested t there might be intramolecular interactions between the C terminus and the central core domain (Hupp al., 1995 ⊡; Jayaraman and Prives, 1995 ⊡). It has been proposed that this interaction locks the core a conformation that is inactive for DNA binding and that the core is then able to adopt the active form when this tail–core interaction is disrupted by phosphorylation, antibody binding, or deletion (Hupp an Lane, 1994 ⊡; Ko and Prives, 1996 ⊡). In addition, there appears to be a competitive disruption of th interaction by synthetic C-terminal peptides that can activate p53 in *trans* (Hupp et al., 1995 ⊡; Jayaraman and Prives, 1995 ⊡).

The acetylation by p300 of these lysines in the C terminus of p53 leads to a neutralization of positive charges that in turn could disrupt the tail–core interaction and activate p53 DNA binding. To further confirm this hypothesis, we tested the effect of acetylation on the ability of the C-terminal peptide to activate p53 in *trans*. As shown in Figure 6A, the unmodified form of the synthetic peptide derived fro the C terminus strongly activated (up to 7.8-fold) the DNA-binding capability of the full-length p53 (Figure 6B), whereas the acetylation of the C-terminal peptide significantly (~2-fold) reduced its abilit activate p53 in the same assay (Figure 6A and Figure 6B). This result suggests that the acetylated C-terminal peptide has a reduced ability to interact with the core domain and thus is less active in stimulating DNA binding of the full-length p53 in *trans*.

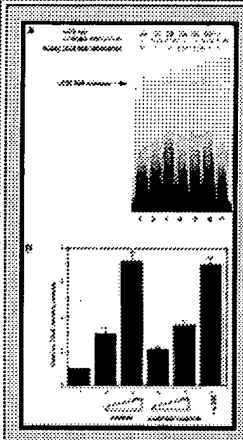

Figure 6. Acetylation of p53 C-Terminal Peptide (364–389) Inhibits Its Ability Tc Stimulate DNA Binding by Full-Length p53

(A) Effects of unmodified versus acetylated peptides on p53 binding to DNA. Human p53 (25 ng) was incubated with ^{32}P-labeled wild-type GADD45 oligonucleotides (0.2 ng) in the absence (lane 1) or in the presence of the indicatec amounts of unmodified (lanes 2, 3, and 6) or acetylated (lanes 4 and 5) p53 C-terminal peptide (364–389). In lane 6, 25 ng of p53 was incubated with the prol in the presence of 0.50 nmol of the p53 C-terminal peptide (364–389) and 10 ng o p300(1195–1810), indicating that contaminating p300(1195–1810) from acetylate p53(364–389) has no effect on p53 DNA binding. Lane 7 contained the oligonucleotide probe and 0.50 nmol of p53 C-terminal peptide (364–389) but no human p53 protein. The arrow indicates the p53/DNA complex.

(B) Quantitation of the p53 DNA binding data from panel A. Data were quantitate by phosphorimaging using Image-Quant software, and representive results depict the average of three experiments with standard deviations indicated.

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Interestingly, previous studies indicated that substitution of any of the five lysines (K370, K372, K373 K381, and K382) with alanine dramatically reduces the ability of the synthetic C-terminal peptide to activate p53 in *trans* (Hupp et al., 1995 ⊡). This is completely consistent with our discovery that acetylation of these five lysines reduces the ability of the C-terminal peptide to activate p53 in *trans*.

Discussion

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p300/CBP has emerged as a transcriptional coactivator for a broad group of cellular DNA-binding regulatory proteins (reviewed in Shikama et al., 1997), including the tumor suppressor p53 (Gu et a 1997; Lill et al., 1997). Following our earlier demonstration that p53 functions synergistically wi p300/CBP, we show here: (i) that p53 is a substrate for the acetyltransferase activity of p300; (ii) that acetylation of p53 is restricted to the C terminus and activates the latent sequence-specific DNA-bindi activity of p53; (iii) that p53 is acetylated in vivo, as well as in vitro; and (iv) that residues in the acetylation site are evolutionary conserved. These results are especially relevant to the in vivo transcription function of p53 and, since sequence-specific transcriptional activation by p53 correlates v with its ability to suppress cell proliferation, to the associated effect on cell growth. Our discovery is a of special interest in relation to current models regarding the mechanism of transcriptional activation b number of coactivators (including p300) with histone acetyltransferase activity, the assumption having been that histone modifications play the major (if not exclusive) role in the function of these coactivat

Activation of p53 by Acetylation

Transactivation by p53 is tightly regulated in vivo and correlates well with its binding to cognate DNA sequence elements (El-Deiry et al., 1992; Funk et al., 1992; Hansen and Oren, 1997). Most oncogenic mutants of p53 have lost their ability to function effectively in a sequence-specific DNA-binding manner, suggesting that these activities of p53 are critical for its tumor suppressor funct Furthermore, considerable evidence indicates that posttranslational modifications of the carboxyl termi of p53 play a key role in converting p53 from an inert to an active form for DNA binding (Ko and Priv 1996).

The specific mechanisms involved in p53 latency and activation after posttranslational modification rer unclear. According to the allosteric model (Hupp et al., 1992 ⊡ Hupp et al., 1995 ⊡; Jayaraman and Prives, 1995 ⊡), the C-terminal tail of p53 interacts with the core DNA-binding domain and locks the DNA-binding domain into an inactive conformation. Significant in this regard is our demonstration of p300-mediated acetylation of specific lysine residues in the C terminus of p53, with a consequent activation of DNA binding. The acetylation-mediated neutralization of positive charge could disrupt interactions between the C-terminal domain and the core domain, thus allowing the DNA-binding dom to adopt an active conformation (Figure 7). This model is further substantiated by our observation that acetylation of the C-terminal peptide abrogates its ability to activate DNA binding by the full-length p5 *trans*. As suggested in Figure 7, the unmodified C-terminal peptide could activate p53 simply by competitive disruption of the core–tail interaction, whereas the acetylated form that lacks the positive charges would also lack the ability to fully activate p53 in *trans*. Interestingly, the proposed role of acetylation in reversing the p53 autoinhibitory mechanism is analogous to the role of phosphorylation i blocking the function of autoinhibitory domains in several kinases (Engel et al., 1995 ⊡; Mohammadi al., 1996 ⊡).

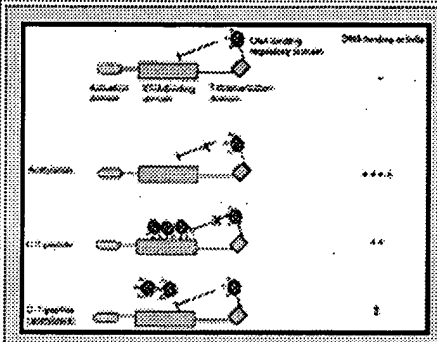

Figure 7. A Model for Activation of the Latent DNA-Binding Activity of p53 by Acetylation

Full-length p53 exists in a latent DNA-binding form as result of the tail–core interactions, as suggested by several studies (Hupp et al., 1992 ⊡; Hupp et al., 1995 ⊡; Jayaraman and Prives, 1995 ⊡). Acetylation of lysine residues in the C-terminal region results in neutralization of positive charges and leads to the disruption of interactions between the C-terminal domain and the core domain, thus allowing the DNA-binding domain to adopt an active conformation. Competitive disruption of this tail–core interaction b synthetic C-terminal peptides can also activate the DNA binding of the full-length p53 (Hupp et al., 1995 ⊡; Jayaraman and Prives, 19 ⊡). However, when the positive charges in the C-terminal peptides are neutralized by acetylation, the acetylated peptide loses the abili to activate p53 DNA binding in *trans*.

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The ability to activate the p53-mediated transcriptional function both in vitro and in vivo, by interactio either with monoclonal antibody PAb421 (Hupp et al., 1995) or with C-terminal peptides (Selivano et al., 1997), underscores the rate-limiting nature of the sequence-specific DNA binding in p53 activation. These findings also suggest the presence of a cellular pool of latent p53 that can be activate posttranslationally. Serine phosphorylation of the carboxyl terminus has also been shown to stimulate t DNA binding of p53 in vitro (Hupp et al., 1992; Meek, 1994). However, elimination of one phosphorylation site (serine 386 in mouse p53 or serine 392 in human p53) by alanine substitution had significant influence on p53-dependent transactivation in cells (Fiscella et al., 1994; Hall et al., 199€), raising the question of whether this type of phosporylation is functional in vivo. It is possible that tl effects of other activation pathways (e.g., acetylation) may mask this effect in vivo.

Compared with antibody PAb421 binding, addition of C-terminal peptides, and phosphorylation, activation of p53 by acetylation may be more physiologically relevant. This is supported by our previoı observations that p300/CBP strongly interacts with p53, both in vivo and in vitro, and potentiates p53-mediated transcriptional activation in cells (Gu et al., 1997; Lill et al., 1997). Furthermore, t present study shows that residues in the acetylation site are evolutionarily conserved and, importantly, p53 is also specifically acetylated in vivo. Future studies must involve the development of methods to confirm the in vivo-acetylated lysine positions and to analyze the regulation of p53 acetylation by CBP/p300 in the cell. Interestingly, many deacetylase inhibitors, including sodium butyrate, trichostati and trapoxin, induce cell growth repression, transformation reversion, and apoptosis (Kijima et al., 19 ; Yoshida et al., 1995; Buckley et al., 1996). These phenotypes are very similar to the effects o p53 activation in the cell, making it interesting to find out whether these histone deacetylase inhibitors also inhibit p53 deacetylation and whether some histone deacetylases can directly deacetylate p53.

In addition, the proportion of the whole p53 protein population that is acetylated in normal cells and h the acetylation of p53 is regulated, particularly in response to specific stimuli (e.g., UV), remain unknc Since an antitumor agent, epoposide, can activate p53 and induce apoptosis in human testicular tumor switching the latent form into an active form of p53 (Chresta and Hickman, 1996; Lutzker and Levi 1996), the present studies suggest that it may be useful to develop an activation strategy based on tl stimulation of p53 acetylation.

p300/CBP: HAT versus FAT Functions

As first reported for the yeast coactivator GCN5 (Kuo et al., 1996), mammalian coactivators p300/CBP (Bannister and Kouzarides, 1996; Ogryzko et al., 1996), P/CAF (Yang et al., 1996 and $TAF_{II}250$ (Mizzen et al., 1996) were shown to possess intrinsic HAT activities. Similarly, varic yeast and mammalian corepressors were implicated as, or shown to interact with, proteins that have intrinsic histone deacetylase activities (Alland et al., 1997; Hassig et al., 1997; Heinzel et al., 19 ; Kadosh and Struhl, 1997; Laherty et al., 1997; Nagy et al., 1997; Zhang et al., 1997). Histone acetylation can result in nucleosome destabilization and chromatin remodeling (Turner, 1993 Brownell and Allis, 1996; Wolffe and Pruss, 1996), which is likely important for accessibility anc stable binding of transcription factors to cognate DNA-binding sites within the chromatin templates, a in some cases, histone acetylation has been correlated with increased levels of transcription (Van Lint al., 1996; Chen et al., 1997). Hence, it has been proposed that transcriptional activation by sequence-specific activators may involve recruitment and function of histone acetyltransferases, while transcriptional repression by sequence-specific repressors may be mediated by the recruitment of histo deacetylases (Pazin and Kadonaga, 1997). Consistent with this view, mutations of the HAT domain have been shown to inactivate the coactivator function of GCN5 in yeast (Candau et al., 1997). However, there is no direct evidence that histone acetylation and deacetylation events are either causal related to, or the exclusive function of, the cognate coactivators and corepressors.

In support of the possibility of alternate functions for coactivators with acetyltransferase (or deacetyla activities is our demonstration that p300 has a potent transcription factor acetyltransferase (FAT) acti in addition to the HAT activity, and that p300-mediated acetylation of p53 can dramatically increase t latent DNA-binding activity. The direct effect of the p300 FAT function is clear, since the binding stuc were done in vitro with recombinant proteins, and the observations that p53 is acetylated in vivo and t sequence-specific DNA binding of p53 is limiting for p53-mediated transcriptional activation suggest t a similar p300 FAT function is operative in vivo. The possibility of cofactor FAT activities is further supported by the observation that other general transcriptional factors and cofactors can be acetylated p300 in vitro (W. G. and R. G. R., unpublished data).

Our present study adds an important new perspective to our current understanding of the molecular mechanism of acetyltransferase/coactivator functions in transcriptional activation by implicating a FA function that may operate in place of or, more likely, in addition to the HAT function. Thus, acetyltransferase-containing coactivators may be recruited by (interact with) sequence-specific activat for acetylation of either the activators themselves (e.g., p53) or the general transcription machinery (e. TFIIE, TFIIF, and PC4; W. G. and R. G. R., unpublished data), or both, with consequent transcriptior activation. At the same time, the recruitment of acetyltransferases to the promoter region could also induce local nucleosome modifications by histone acetylation, facilitating stable binding of the activato or general transcriptional factors to the nucleosome template prior to transcription initiation. These mechanisms are especially applicable to HAT/FAT coactivators that are targeted to promoters either b direct interactions with site-specific DNA-binding proteins (p300/CBP, $TAF_{II}250$) or through interact with associated factors (P/CAF, GCN5) that in turn recognize site-specific DNA-binding proteins.

Interestingly, several studies have indicated that transiently transfected templates are not efficiently packaged into chromatin (Archer et al., 1992 ⊡; Van Lint et al., 1996 ⊡; Pazin and Kadonaga, 1997 and that upregulation of histone acetylation with deacetylase inhibitors can result in a marked increase transcription of integrated templates with little or no effect on transiently transfected templates (Van L et al., 1996 ⊡; Chen et al., 1997 ⊡). In this regard, synergism between p300/CBP and interacting activators (including p53) is mainly evident on transiently transfected templates (Gu et al., 1997 ⊡; Lil al., 1997 ⊡; reviewed in Shikama et al., 1997 ⊡). Along with our more direct demonstration of functi FAT activities, these results further support our hypothesis that coactivator-mediated acetylation of nonhistone regulatory proteins may be important in transcriptional activation.

Furthermore, although a major function of many sequence-specific activators may be to recruit acetyltransferase-containing coactivators for utilization of the acetyltransferase activities per se, other functions, such as direct interactions that result in recruitment of the general transcriptional machinery, also possible for both activators (reviewed in Ptashne and Gann, 1997 ⊡) and acetyltransferase-contai coactivators (Kwok et al., 1994 ⊡; Nakajima et al., 1997 ⊡; reviewed in Shikama et al., 1997 ⊡). In t case of p53, secondary activator functions are indicated by the synergistic effect of p53 on activation b CBP, even when the latter is tethered to the promoter by an alternative mechanism (Gu et al., 1997 ⊡), and may reflect the p53–TAF interactions implicated in target promoter activation by p53 in vitro (Lu Levine, 1995 ⊡; Thut et al., 1995 ⊡). Taken together, these studies and the work reported here supp model in which both DNA-binding activators and interacting acetyltransferase/coactivators may have multiple functions in a multistep transcriptional activation pathway. A speculative comprehensive mod for p53 would be that acetylation by p300 leads to joint activator/ coactivator recruitment to the promoter, both through enhanced p53 DNA-binding capability and through persistent p53–p300 interactions, and that subsequent functions of both p300 (e.g., histone acetylation) and p53 (e.g., TAF interactions) are then possible.

In conclusion, we have provided an example of a nonhistone regulatory protein, p53, which can be acetylated both in vitro and in vivo, and we have shown that acetylation of p53 by its coactivator p300 dramatically activates its biochemical function. This study clearly suggests a potential pathway for activation of p53 in vivo and predicts that other cellular regulatory proteins can be modified and functionally regulated in a similar manner. It further supports the views that protein acetylation and deacetylation are fundamental regulatory mechanisms in transcriptional regulation.

Experimental Procedures

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Plasmids and Recombinant Proteins

For production of GST fusion proteins, DNA sequences corresponding to the indicated regions of hun p53 were amplified by PCR and subcloned into pGEX-2T (Pharmacia). GST fusion proteins were expressed in E. coli, extracted with buffer BC500 (20 mM Tris–HCl [pH 8.0], 0.5 mM EDTA, 500 ml KCl, 20% glycerol, 1 mM DTT, and 0.5 mM PMSF) containing 1% NP-40, and purified on glutathione-Sepharose (Pharmacia). The Flag-p53 and Flag-p300(1195–1810) proteins were expresse BL21 (Lys) cells at room temperature and purified on an M2 agarose affinity column as indicated previously (Chiang and Roeder, 1993 ⊡; Gu et al., 1997 ⊡). All proteins were dialyzed in buffer BC1 (20 mM Tris–HCl [pH 8.0], 0.5 mM EDTA, 100 mM KCl, 20% glycerol, 0.5 mM DTT, and 0.5 mM PMSF) before storage at -80°C.

Protein Acetyltransferase Assays

Protein acetyltransferase assays were performed essentially as described (Ogryzko et al., 1996 ⊡) with some modifications. In the standard assay, 30 μl reactions contained 50 mM HEPES (pH 8.0), 10% glycerol, 1 mM DTT, 1 mM PMSF, 10 mM sodium butyrate, 1 μl of [^{14}C]-acetyl-CoA (55 mci/mmol, Amersham), 1 μg of highly purified substrate proteins or 2.5 μg of GST fusion proteins, and 100 ng of p300(1195–1810) and were incubated at 30°C for 1 hr. For filter binding assays, the reaction mixture spotted onto Whatman P-81 phosphocellulose filter paper. The filter paper was air-dried for 2–5 min a washed with 0.2 M sodium carbonate buffer (pH 9.2) at room temperature with five changes of the bu for a total of 30 min. For electrophoretic assays, the reaction mixture was subjected to SDS–PAGE ge and autoradiography. Gels containing [^{14}C]-acetate-labeled proteins were fixed with 10% glacial aceti acid and 40% methanol for 1 hr and were enhanced by impregnating with a commercial fluorography enhancing solution (Amplify, Amersham) for 30 min. Gels were then dried and autoradiography was performed at -70°C for 1–3 days. For the preparation of acetyl-p53 and acetyl-p53(364–389) for EMS [^{14}C]-acetyl-CoA was replaced with unlabeled acetyl-CoA (Pharmacia) in the standard assay.

Mapping p53 Acetylation Sites

Synthetic peptides corresponding to the C terminus of p53 (residues 364–389) were synthesized by th protein/DNA technology center of the Rockefeller University and purified to 95% purity by HPLC. Peptides were incubated with [^{14}C]-acetyl-CoA and p300 at 30°C for 2 hr. After incubation, the acetylated peptides were separated from contaminant p300 after the reaction by passage through a Microcon 10 (Amicon). The peptides were then subjected to standard N-terminal peptide microsequenceing analyses with 50% of each cycle used for amino acid identification and 25% for radioactivity determination by liquid scintillation counting.

Cell Transfection, Labeling, and Immunopurification

SaoS-2 cells (2×10^6) were transfected by calcium phosphate precipitation on a 10-cm plate essentiall previously described (Gu et al., 1994 ⊡) with minor modifications. p53 or p53Δ370 plasmids (5 μg) (Crook et al., 1994 ⊡) with carrier plasmid pcDNA3 (15 μg) were used for transfections on each plat Thirty hours after transfection, the cells were transferred to the same DMEM medium containing 1 mc [^{3}H]-sodium acetate (2–10 Ci/mmol, about 100 mCi total) (Amersham) for 1 hr before lysis. All steps the immunoprecipitation were carried out on ice or in the cold room. Cells were washed twice with co phosphate-buffered saline and lysed in lysis buffer with freshly added protease inhibitors (50 mM HEPES-KOH [pH 8.0], 150 mM NaCl, 1% Triton X-100, 0.2% Sarkosyl, 10% glycerol, 0.5 mM DT mM NaF, 1 mM Na_3VO_4, 10 mM sodium butyrate, 0.5 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 0.5 mM PMSF). The lysate was centrifuged twice at 17,000 g for 30 min at 4°C. Supernatants collected from 10 plates were immunoprecipitated with the indicated antibodies, which h been cross-linked to agarose beads, for 4 hr at 4°C. The beads were washed five times with 1 ml of lys buffer. Immunopurified proteins were solubilized with SDS–PAGE sample buffer and resolved on 10% SDS–PAGE gels. Gels containing [^{3}H]-acetate-labeled p53 were fixed with 10% glacial acetic acid an 40% methanol for 1 hr and enhanced by impregnating with a commercial fluorography enhancing solu (Amplify, Amersham) for 30 min. Gels were then dried and subjected to autoradiography at -70°C for 10–14 days.

Immunoblotting

Proteins were resolved on 10% SDS–PAGE gels, followed by semidry transfer to nitrocellulose memb for 1 hr at room temperature. After incubation with the first antibody for 2 hr at room temperature, blc were subsequently incubated with secondary antibody and visualized by ECL as suggested by the manufacturer (Amersham).

Electrophoretic Mobility Shift Assay

EMSA was carried out essentially as described (Jayaraman and Prives, 1995 ⊡) with some modificatic Sequences of the oligonucleotides containing either wild-type or mutant p53 DNA-binding sequence a as follows: GADD45 wild type, TACAGAACATGTC TAAGCATGCTGGGG; GADD45 mutant, TACAGAATCGCTCTAAG CATGCTGGGG. The DNA binding reactions (20 μl) contained 20 mM Tris–HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA, 10% glycerol, 10 mM sodium butyrate, 0.5 mg/ml BSA, 100 ng poly(dI-dC), and proteins as indicated. Reaction mixtures w preincubated at room temperature for 20 min before a ^{32}P-labeled probe DNA (0.2 ng) was added and further incubated at room temperature for 20 min. Each reaction mixture was then loaded onto a nativ 4% polyacrylamide gel (acrylamide:Bis, 50:1) containing 0.5 × TBE and electrophoresed in 0.25 × TB 180–220 V for 3 hr at 4°C. All procedures were basically the same for the analyses of activation of p5 DNA binding by synthetic peptides, except that the preincubation was performed at 30°C for 30 min before the probe was added. In the case of supershift assays, the indicated monoclonal antibodies (200 were added to reaction mixtures during preincubation.

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Footnotes

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